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Unveiling new microbial eukaryotes in the surface ocean

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A decade after molecular techniques were used to discover novel bacteria and archaea in the oceans, the same approach has revealed a wealth of new marine eukaryotic microbes. The approach has been particularly successful with the smallest eukaryotes, where morphological and culture approaches frequently fail. Analysis of samples from the surface ocean, the most accessible and supposedly well-known oceanic region, reveals novel eukaryotic diversity at all different levels: from the highest taxonomic rank to the lowest microdiverse clusters. Moreover, marine eukaryotic assemblages show a large diversity with members belonging to many different lineages. The implication of this large and novel eukaryotic diversity for biodiversity surveys and ecosystem functioning opens new avenues for future research.

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Introduction

Microorganisms are known to play essential roles in natural systems. In the surface oceans they are present in large abundances, account for a significant share of planktonic biomass, and are central in biogeochemical cycles. Yet, they are a heterogeneous collection of organisms with a substantial diversity of form, size, life style, and phylogenetic affiliation, and they are pooled together only because of their invisibility to the naked eye [1•]. Many microorganisms, especially the smallest ones, cannot be identified under the microscope, because they lack conspicuous morphological features. This is well known in prokaryotes, which have only a handful of possible morphologies. Only after molecular surveys retrieving SSU rDNA sequences directly from the environment were carried out, the phylogenetic affiliation of marine prokaryotes was known. The first studies on the molecular diversity of marine bacteria [2] and archaea [3] unveiled a substantial amount of new diversity and revealed that most microorganisms available in pure culture were not dominant in the marine plankton. Microbial eukaryotes, in turn, were not considered in these early approaches, despite the fact that the smaller eukaryotes were also difficult to be identified morphologically. In most conventional studies of marine plankton, these organisms were usually lumped in a black box labeled 'small flagellates'. The growing recognition of the importance of these minute eukaryotes as primary producers, bacterial grazers, and parasites paralleled the interest of identifying the species of these probably very diverse assemblages. The first molecular surveys of marine eukaryotes confirmed the existence of significant novel diversity within the protistan world [4••,5••,6••]. This approach has benefited from recent efforts to build a robust framework of eukaryotic evolution within which environmental sequences can be placed. Virtually all eukaryotic organisms can be grouped into a few supergroups. Each one is composed by distinct lineages (most are protists) that are held together by phylogenetic signatures and some ultrastructural characters [7,8••,9]. For instance, the supergroup opisthokonta includes metazoans, fungi, and choanoflagellates. Here we analyze and summarize the novel diversity of marine microbial eukaryotes as revealed by molecular studies in the surface of the oceans.

A new window into protistan diversity in the sea

Introduction of molecular tools in microbial ecology has become the key to access the phylogenetic and functional diversity of marine microbes [10]. The basis is to extract total DNA from a community, to amplify a marker gene (18S rDNA in eukaryotes) by PCR, and to clone and sequence the PCR products for phylogenetic identification. The first molecular surveys of marine eukaryotes targeted the smallest cells (picoeukaryotes, $\leq$3 μm in size) from surface [4••,6••] and deep [5••] oceanic samples. Extreme environments, such as anoxic water column and sediments [11,12] and hydrothermal vents [13] were inspected soon after, in the search for the limits of eukaryotic life and the most divergent and ancient lineages. After the sequencing effort, specific oligonucleotide probes could be designed and applied through fluorescent *in situ* hybridization (FISH) to visualize the target cells in natural samples and to determine their distribution and abundance. This has been done for groups having both cultured [14] and uncultured [15,16••,17] representatives. It is in the latter case where FISH exploits its full potential, allowing to put a face (cell size, rough shape, chlorophyll presence) to novel lineages

Table 1

Affiliation of 18S rDNA sequences from surface picoplankton in the coast (23 libraries and 1349 clones) and open sea (12 libraries and 826 clones) from studies that reported the clonal distribution among phylogenetic groups

Supergroup	First rank	Second rank	*n*	% clones	Habitat	Novelty[a]
Archaeplastida	Chloroplastida	Prasinophytae	261	15.0	Coast	Low
		Chlorophyta	2	0.1		–
Chromalveolata	Alveolates	Ciliophora	137	4.9	Coast	Medium
		Dinozoa	173	5.3		Low*
		MA-I	363	14.6		High
		MA-II	417	17.6	Coast	High
	Stramenopiles	Bicosoecida	20	0.4		Low
		Bolidophyceae	21	0.4		Low
		Chrysophyceae	65	2.7		Medium
		Diatoms	27	2.5		Low*
		Dictyochophyceae	25	1.1		Low
		Eustigmatales	2	0.4		–
		Labyrinthulids	14	0.5		Medium
		MASTs	237	13.4	Offshore	High
		Oomycetes	8	0.2		–
		Pelagophyceae	12	1.0	Offshore	Low
		Pirsonia	3	0.1		–
	Basal groups	Cryptophyceae	64	2.4	Coast	Low*
		Haptophyta	53	4.5	Offshore	Low*
		Katablepharis	6	0.2		–
		Telonema	14	0.4		Medium
Excavata	Euglenozoa	Kinetoplastea	1	0.0		–
Opisthokonta	Choanoflagellates		16	1.0		Medium
	Fungi		16	0.8		High
Rhizaria	Cercozoa		53	2.6	Coast	Medium
	Chlorarachniophyte-like		11	0.3		High
	Radiolaria		90	5.6	Offshore	High
Not assigned	Apusomonads		11	0.2		Low
	Picobiliphyta		24	0.9		High
	Inserta sedis		29	0.7		High

Data derive from Pacific, Atlantic, Indian, and Southern Oceans, and the Mediterranean and North Seas [4••,6••,22–27]. The number of clones, the average contribution of each group (normalized by the number of clones in each system), the coast-offshore trend, and a rough indication of the level of novel diversity detected within the group are shown.

[a] Low: most clones are ≥98% similar to cultured relatives (* indicates a significant presence of more divergent clones); medium: most clones are 92–98% similar to cultured relatives; high: most clones are <92% similar to cultured relatives.

detected by environmental sequencing. Finally, the metagenomic approach retrieves the full gene content of natural assemblages [18,19], providing a picture of microbial diversity independent both from culturing and PCR biases. This is done by direct cloning of small or large fragments of environmental DNA for later sequencing. Although metagenomic studies on marine microbes have so far targeted mostly the prokaryotic size fraction [20•], they contain a few 18S rDNA sequences [15]. A metagenomic study including picoeukaryotes has been recently published [21].

Here we analyze the 18S rDNA sequences of marine picoeukaryotes derived from the euphotic marine zone, the well-oxygenated surface skin of the ocean that is also most reactive to biogeochemical cycles. We examined 35 PCR-based libraries prepared with coastal and open sea samples from the Pacific [6••,22], Atlantic [23], Indian [24], and Southern Oceans [4••], and the Mediterranean [4••,25] and North Seas [26,27]. These are the libraries where enough information was provided to calculate relative clonal abundance, and yield information comparable to other studies [28–30]. The 2175 clones are distributed among all eukaryotic supergroups, with the exception of amoebozoa (Table 1). Alveolates (43% of clones), stramenopiles (23%), and prasinophytes (15%) are the better-represented groups. Some seem more important in coastal areas (cercozoans, ciliates, cryptophytes, alveolates-II, and prasinophytes), and others in the open sea (MASTs, pelagophytes, haptophytes, and radiolarians). Even in this supposedly well-known marine habitat, the most accessible and well studied, substantial novel diversity is revealed at different phylogenetic scales: high-rank novelty (sequences outside supergroups), intermediate-rank novelty (novel lineages within supergroups), and low-rank novelty (sequences close but not identical to characterized organisms, putatively representing new species, genera, orders, or families).

High-rank novel diversity: more than the six supergroups?

The first molecular surveys of marine protists claimed the discovery of novel groups that deserved the highest taxonomic rank, which could not be placed within any of the eukaryotic supergroups [5••,11,12]. Many of these sequences derived from anoxic systems. However, it was soon shown that some of these highly divergent groups were unsupported because of the presence of undetected chimeras, misplacement of fast evolving lineages, and incomplete representation of cultured strains [31••,32,33]. However, several sequences still form robust and deep clades and thus remain as candidates for novel high-rank taxonomic groups. They are found at low clonal abundance so, probably, they are not very important ecologically. Instead, their interest is that they might represent different pathways in eukaryotic evolution. Perhaps the best example is the picobiliphytes, a novel phytoplanktonic class that is not close to any supergroup [17]. Picobiliphyte cells are 3–4 μm in size, putatively contain a plastid with phycobilin pigments, and can be locally abundant [15]. Other high-rank groups await careful scrutiny, because each library often yields a few sequences impossible to be classified to a given supergroup. Twenty-nine clones of the overview presented here could not be assigned to a given supergroup (Table 1). The similarity of these sequences is often below 75% to any other known 18S rDNA. Some of them appear in more than one system, thus excluding the possibility of being chimeras. Exhaustive phylogenetic reconstructions, renovated culturing efforts, and additional sequences from single cells are needed to understand the biological nature of these putative high-rank lineages.

Intermediate-rank novel diversity: novel eukaryotic lineages

A large number of sequences form clades that affiliate to a given eukaryotic supergroup but without a clear affiliation to any defined group. Among these, the marine alveolates (MAs) and marine stramenopiles (MASTs) are particularly interesting because they appear in virtually all marine surveys. MAs are divided into two main groups, MA-I and MA-II, which form robust lineages equivalent to the other alveolate groups. Their placement is still unresolved, because SSU analysis places them closer to dinoflagellates [34], whereas a recent LSU tree places them closer to perkinsids [21]. The sequence diversity contained in the two groups is huge, with at least 5 clades within MA-I and up to 16 clades within MA-II [35•]. Besides their genetic diversity, these groups appear at a very high clonal abundance, 14.6% for MA-I and 17.6% for MA-II. Soon after the description of MAs, *Amoebophrya* (a dinoflagellate parasite) was sequenced and affiliated to MA-II [36]. Additional sequences of parasites have been later published within both MA-I and MA-II [34,37,38]. Therefore, it has been proposed that the whole assemblage is composed of parasites of marine organisms. Perhaps, the specific interaction with different hosts could explain their large genetic diversity. Their considerable clonal abundance and diversity suggests an important role for parasitism as a trophic relationship in the open sea.

MASTs form more than 10 clades at the basal part of the stramenopiles [39•], where all protists are heterotrophic, including free-living phagotrophic flagellates (bicosoecids), parasites (*Blastocystis*), or osmotrophs (oomycetes and labyrinthulids). MASTs are rather abundant (13.4% of clones), and a few clades (MAST-1, MAST-3, MAST-4, and MAST-7) account for most sequences (the other clades have lower clonal abundances or are specific of anoxic systems). The heterotrophic nature of MASTs, first suspected by their phylogenetic placement, was confirmed by FISH for clade-1, clade-2, and clade-4 [16••]. These are small protists (2–8 μm in size), able to grow in the dark and to ingest bacteria. These MAST cells are widely distributed and account for a significant fraction of heterotrophic flagellates globally. One group in particular, MAST-4, is found in all samples (except the polar ones) as a very small protist (2–3 μm in size), its abundance averages 130 cells ml^{-1}, and accounts for 9% of heterotrophic flagellates. Overall, these results reveal that still-uncultured groups can be dominant in the oceans and highlight the ecological relevance of the novel diversity detected by the molecular approach.

Low-rank novel diversity: known lineages are more diverse than thought

This is represented by sequences that clearly affiliate to a given lineage but are not identical to any characterized protist. Low-rank novel diversity is extensive, because environmental sequences are identical to cultured strains only in a few cases. For instance, only 11 out of 510 partial sequences retrieved from the Indian Ocean [24] were identical (over 800–900 bp) to cultured strains: *Caecitellus parvulus*, *Micromonas pusilla*, and *Ostreococcus* RCC 143. This list would include *Bathycoccus prasinos*, *Amastigomonas debruynei*, *Gymnodinium* sp., and *Pelagomonas calceolata* if 1–2 mismatches were accepted (another 21 clones). So, between 94 and 98% of the sequences retrieved from this marine system represent new diversity not explained by cultured protists.

Obviously, there are degrees of novelty at this lower phylogenetic level. Thus, prasinophytes show the best correspondence between molecular and culturing approaches, and the 18S rDNA sequences from the field and cultures are identical or very close [40]. Other important groups often represented by environmental sequences closely related to cultured strains are the bicosoecids, bolidophytes, dinoflagellates, and pelagophytes. These closely related sequences most probably

identify the same cultured strain in the environment, closely related species, or ecotypes of the same species.

Other groups contain a much larger level of diversity. So, important marine groups such as the ciliates, choanoflagellates, chrysophytes, cryptophytes, diatoms, and haptophytes are represented by environmental sequences that can range from 94 to 100% sequence similarlity to cultured relatives. This implies that some novel clades exist within all these groups, increasing substantially the diversity they contain. For instance, up to three novel clades have been identified within the choanoflagellates, and three novel clades within chrysophytes after analyzing marine sequences (del Campo, Massana, unpublished).

A substantial fraction of clones from the open sea affiliate with the radiolarians (10% on average). This is surprising, because the radiolarian species known so far are rather large (typically around 100 μm) and most possess mineralized skeletons. Marine radiolarian sequences are diverse, generally highly distant to sequenced protists, and form at least five clades, two related to acanthareans, one to polycystinea, and two to taxopodida [23]. Whereas it is known that many described radiolarian species have not been sequenced, given the difficulty of their isolation, it is not clear if these would explain the sequences found in environmental surveys. The existence of these diverse radiolarian sequences from the picoplankton remains as an intriguing enigma.

Microdiversity of natural assemblages

Prokaryotic diversity is normally structured in clades containing highly related but seldom identical sequences [41,42]. The evolutionary and ecological meaning of this microdiversity is not well understood, though it has been proposed that it is a consequence of the asexual mode of prokaryotic reproduction together with ecological factors [43]. Microdiverse clusters would exist because of neutral mutations (also in the 18S rDNA) during asexual divisions, so that all the members would occupy the same ecological niche. When one of these members acquires a selective advantage, periodic selective sweeps would purge all variability within the cluster. Thus, current microdiverse clusters would exist due to the accumulation of neutral mutations since the last selective sweep [44]. It is not clear whether this scenario also holds for microbial eukaryotes. First, it has to be demonstrated that microbial eukaryotes show microdiversity in nature. Some data suggest that this may be the case, at least for some groups such as MASTs [39•] and MAs [35•]. Second, even though cell division in microbial eukaryotes is mostly asexual, it is known (at least for some groups) that sexual events also occur, and these would certainly impact the genetic structure of populations by making populations more homogeneous. It is improbable that sexually compatible organisms show any variation within the 18S rDNA. Mating experiments with related diatom strains showing some genetic structure reveal that only those with an identical 18S rDNA are sexually compatible [45••]. If this applies to other marine populations, then each different 18S rDNA sequence, even with a single base pair difference, would mean an independent and evolutionarily isolated lineage, increasing protist diversity enormously. Thus, the actual microdiversity structure within particular protistan lineages deserves a better study, together with studies to unravel the sexual nature of marine protists.

Conclusions

The analysis of the 18S rDNA sequences retrieved from the sea reveals that marine protists are very diverse, increasing substantially the known amount of diversity within the eukaryotic tree of life. Current parametric and nonparametric estimates of protistan richness [46] indicate that hundreds to thousands of distinct protistan taxa can coexist in a single marine sample [25,28,29]. Most probably, microorganisms do not deviate from the trend of increasing number of species with decreasing individual size [47,48]. As shown here, the relatively low number of microbial species actually described is largely because of the under-representation of microbial diversity in culture collections. The availability of powerful and relatively cheap sequencing techniques will be essential to determine the dimensions of such diversity.

Another interesting point is that the increase in marine eukaryotic diversity occurs at almost all possible phylogenetic scales. Thus, putative high-rank groups occur, novel clades within supergroups have been identified, and novel diversity is detected within all known lineages, from closely new species, genera, families, or orders. The challenge is to retrieve in culture the organisms responsible for such sequences and to determine their trophic role and ecological function.

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